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The Polyamine Oxidase Inhibitor MDL-72,527 Selectively Induces Apoptosis of Transformed Hematopoietic Cells through Lysosomotropic Effects[1]

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ABSTRACT

Polyamine oxidase functions in the polyamine catabolic pathway, converting N^1-acetyl-spermidine and -spermine into putrescine (Put) and spermidine (Spd), respectively, thereby facilitating homeostasis of intracellular polyamine pools. Inhibition of polyamine oxidase in hematopoietic cells by a specific inhibitor, N, N'-bis(2,3-butadienyl)-1,4-butanediamine (MDL-72,527), reduces the levels of Put and Spd and induces the accumulation of N^1-acetylated Spd. Although previously thought to be relatively nontoxic, we now report that this inhibitor overrides survival factors to induce cell death of several immortal and malignant murine and human hematopoietic cells, but not of primary myeloid progenitors. Cells treated with MDL-72,527 displayed biochemical changes typical of apoptosis, and cell death was associated with the down-regulation of the antiapoptotic protein Bcl-X$_L$. However, enforced overexpression of Bcl-X$_L$, or treatment with the universal caspase inhibitor zVAD-fmk, failed to block MDL-72,527-induced apoptosis in these hematopoietic cells. Despite decreases in Put and Spd pools, MDL-72,527-induced apoptosis was not blocked by cotreatment with exogenous Put or Spd, nor was it influenced by overexpression or inhibition of the polyamine biosynthetic enzyme ornithine decarboxylase. Significantly, MDL-72,527-induced apoptosis was associated with the rapid formation of numerous lysosomally derived vacuoles. Malignant leukemia cells were variably sensitive to the lysosomotropic effects of MDL-72,527, yet pretreatment with the ornithine decarboxylase inhibitor L-α-difluoromethylornithine sensitized all of these leukemia cells to the deleterious effects of the inhibitor by stimulating its intracellular accumulation. The lysosomotropic nature of select polyamine analogues may, thus, provide a novel chemotherapeutic strategy to selectively induce apoptosis of malignant hematopoietic cells.

INTRODUCTION

Put[3] and the polyamines Spm and Spd have been implicated as key regulators of many cellular processes including transcription, translation, replication, and the function of ion pores (for review see Refs. 1 and 2). Polyamine levels in cells are tightly controlled by well-characterized synthetic and catabolic pathways and by an active transport system. Metabolism is regulated by the biosynthetic enzymes ODC and SAMDC and by the catabolic enzyme SSAT. The N^1-acetylated-Spd and -Spm products of SSAT are in turn oxidized by an as yet uncloned intracellular oxidase, PAO, which recoups Put and Spd as products, but also generates H$_2$O$_2$ as a byproduct. Thus, the SSAT/PAO pathway participates in lowering intracellular polyamine pools by catabolism and by promoting their excretion out of the cell in the form of acetylated metabolites. Viewed differently, the pathway also promotes polyamine back-conversion, a process that could help sustain Put and Spd pools during cell growth and stress (1, 2).

Given the numerous possible functions of polyamines, it is perhaps not surprising that disruption of polyamine metabolic pathways has pleiotropic effects, including the induction of cell cycle arrest and/or apoptosis. For example, overexpression of ODC (3), or treatment of cells with polyamine analogues that induce SSAT (4–7) or inhibitors of SAMDC or ODC (3, 8, 9), has been shown to induce growth arrest or apoptosis in several cell types. Thus, there has been considerable interest in using these inhibitors or analogues in both chemotherapeutic and chemopreventative regimens, and several inhibitors have been demonstrated to have potential clinical benefit in these scenarios (2, 7, 9–12).

The ability of polyamine analogues to induce apoptosis, in particular some of those that function to potently induce SSAT (4–6), has been linked to their ability to generate increased levels of reactive oxygen (5, 6). Oxidation of the N^1-acetylated-Spd and -Spm products of SSAT occurs through PAO (13, 14), and inhibition of PAO activity using a very specific polyamine analogue inhibitor, MDL-72,527 (15), reduces reactive oxygen levels, and consequently apoptosis, in cells exposed to SSAT agonists or other polyamine analogues (5, 6). However, MDL-72,527 also has the potential to interfere with polyamine pool homeostasis by preventing back-conversion and excretion, and, thus, this inhibitor may also reduce the availability of Put and Spd for cell growth and/or survival.

In both animal and *in vitro* studies, the PAO inhibitor MDL-72,527 has been reported to be relatively nontoxic (12, 16, 17). Although not found to be efficacious on its own, MDL-72,527 has been proposed as an adjunctive therapy in combination with ODC inhibitors in the treatment of some solid tumors (11, 12, 16). The concept here is that disruption of both the synthetic and catabolic arms of the metabolic cycle markedly depletes pools of endogenous polyamines, thus compromising cell growth and/or survival (13–16). In the present study, we report the rather surprising finding that treatment of immortal or malignant hematopoietic cells with MDL-72,527 overrides the survival functions of both hemopoietins and serum to induce a form of apoptosis that is associated with the rapid formation of large lysosomal vacuoles. Because this response was not observed in primary myeloid progenitors, MDL-72527 may prove useful in antileukemia therapeutic regimens.

MATERIALS AND METHODS

Materials. The PAO inhibitor MDL-72,527 [*N,N'*-bis(2,3-butadienyl)-1,4-butanediamine] (15) was generously provided by Hoechst Marion Roussel, Inc. (Bridgewater, NJ). DFMO was provided by ILEX Oncology, Inc. (San Antonio, TX). Put, Spd, Spm, and chloroquine were purchased from Sigma Chemical Co. Aminoguanidine, acetyl-Spd, acetyl-Spm, and MBTH (*N*-methyl-2-benzothiazolone hydrazone hydrochloride) were from Fluka Chemika (Buchs, Switzerland). zVAD-fmk was from Calbiochem (La Jolla, CA).

Cell Culture. The 32D.3 and FDC-P1 cells used in this study are immortal, IL-3-dependent diploid murine myeloid progenitors cells and were maintained

in RPMI 1640 supplemented with IL-3 (20 units/ml) and 10% dialyzed FBS, as described previously (18, 19). Pools and clones of 32D.3 cells engineered to overexpress human Bcl-2 or murine Bcl-X$_L$ were previously described (20, 21) and were cultured in IL-3 medium containing G418 (0.4 mg/ml). Primary murine myeloid progenitor cells were derived from day 15 fetal livers. Fetal liver cells were cultured in RPMI 1640/10% FBS medium supplemented with IL-3 (20 units/ml), stem cell factor (10 ng/ml; R&D Systems), and IL-6 (10 ng/ml, R&D systems), as described previously (21). L1210, Molt3, HL-60, and U-937 leukemia cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 supplemented with 10% FBS plus 50 μM β-mercaptoethanol and 1% penicillin/streptomycin (Sigma Chemical Co.). All cells were grown in vented 75-cm tissue culture flasks at 37°C with 5% CO$_2$ in a humidified incubator.

Cell cycle distribution was determined using a FACScan (Becton Dickinson), as described previously (18). To address effects of MDL-72,527 on hematopoietic cell viability, cells were treated with the indicated doses of the inhibitor. Effects of this inhibitor on the rates of hematopoietic cell death were assessed by treating logarithmically growing cells twice in replete growth medium at a density of 3–5 $\cdot$ 10^5 cells/ml. Viable cell number was determined using a hemocytometer and trypan blue dye exclusion.

To inhibit ODC enzyme activity, cells in replete medium were incubated with 5 mM DFMO. At this dose of DFMO there are dramatic decreases in Put and Spd pools in 32D.3 cells that leads to arrest in G$_1$, an effect that is reversible by the addition of exogenous Put (19). After 48 h in DFMO, MDL-72527 was added to the cultures and cell viability scored as above. Where indicated, aminoguanidine (50 μM) was added to the cells and even higher concentrations (1 mM) were without effect.

Apoptosis Assays. Exponentially growing 32D.3 myeloid cells were treated with 150 μM MDL-72,527. Cell morphology was analyzed following cytospin and staining with Wright-Geimsa. To monitor DNA integrity, genomic DNA was isolated from 1 $\cdot$ 10^6 cells and analyzed on a 2% agarose gel, as described previously (18, 22). Annexin V assays were carried out according to the manufacturer's instructions using the ApoAlert Annexin V Apoptosis Kit (Clontech, Palo Alto, CA). Annexin V detects phosphatidylserine, which is translocated from the inner to the outer leaflet of cytoplasmic membrane when cells undergo apoptosis (23). Briefly, cells (5 $\cdot$ 10^6) were washed with cold PBS, resuspended in 200 μl of binding buffer, and incubated with 1 μg/ml Annexin V-FITC and PI for 10 min in the dark. Samples were then analyzed by flow cytometry using a single laser emitting excitation at 488 nm. Early apoptotic cells are Annexin V bright and PI low (23).

PAO Assay. PAO activity was determined according to the method of Morgan (24). Briefly, aminoaldehydes formed by oxidation of N^1-acetyl Spm by PAO are trapped with MBTH and interact with ferric chloride to form a blue product, which is monitored by measuring the absorbency at 660 nm. Exponentially growing cultures of 32D.3 cells were treated with various doses of MDL-72,527, and cell extracts were prepared as described (24).

Polyamine and MDL-72,527 Pools. Intracellular polyamines were extracted from snap-frozen cell pellets (10^7 cells/pellet, in duplicate) using 500 μl of 0.6 N perchloric acid, and the samples were dansylated and quantitated

by reverse phase HPLC, as described previously (25, 26). Data were expressed as nmol/mg protein.

Electron Microscopy. Cells were collected by centrifugation, washed in PBS, and fixed using 3% phosphate-buffered glutaraldehyde. Samples were then postfixed with 1% osmium tetroxide, embedded in Spurr, and sectioned. The sections were stained with uranyl acetate and lead citrate and viewed in a JEOL 1200 electron microscope.

Immunoblot Analyses. Preparation of cell extracts and immunoblot analyses were performed as described previously (21). Polyclonal anti-CPP32 (caspase-3 1:1,000) antibody was a gift from Dr. Xiaodong Wang (Southwestern Medical Center, University of Texas, Dallas, TX). Anti-PARP C-2-10 antibody was from Pharmigen (1:1,000). Antibodies for Bcl-2 family proteins were: mouse Bcl-2 (15021, PharMingen, 1:250); mouse Bcl-X (B2260, Transduction Laboratories, 1:250); mouse Bax (13686E, PharMingen, 1:500); and Bad (B36420, Transduction Laboratories, 1:250).

RESULTS

The PAO Inhibitor MDL-72,527 Overrides the Survival Factors IL-3 and Serum to Induce Cell Death of Immortal Myeloid Progenitors. PAO catalyzes the oxidation of N^1-acetylated-Spd and -Spm to Put and Spd, respectively (10). Using MDL-72,527 we, therefore, initially addressed whether PAO activity played an active role in maintaining polyamine pools of hematopoietic cells. Murine IL-3-dependent 32D.3 progenitors provide a useful model system to address the consequences of manipulating polyamine metabolism. In particular, these myeloid cells respond to ODC overexpression or inhibition by undergoing apoptosis or cell cycle arrest, respectively (3, 19). 32D.3 cells were treated with increasing concentrations of MDL-72,527 and analyzed for PAO enzyme activity and polyamine pools (Fig. 1). Because polyamines present in serum could mask potential effects of MDL-72,527, cells were cultured in IL-3 medium with 10% dialyzed serum. At a dose of the inhibitor that suppressed >75% of PAO enzyme activity within 12 h (150 μM; data not shown), there was a marked reduction of both intracellular Put and Spd pools and a significant increase in the levels of the PAO substrate intermediate N^1-acetyl Spd. By contrast, levels of Spm were relatively unaffected by this inhibitor, and we failed to detect N^1-acetyl Spm (Fig. 1 and data not shown). Thus, steady-state pools of at least Put and Spd are compromised by inhibition of PAO in 32D.3 myeloid cells.

A byproduct of the oxidation of acetylated polyamines by PAO is H$_2$O$_2$. Given the deleterious effects of reactive oxygen species as inducers of apoptosis, a reasonable expectation is that treatment of cells with a specific inhibitor of PAO would be beneficial to cell survival, particularly under stressful conditions. To address the consequences of MDL-72,527 on cell growth and survival, we treated
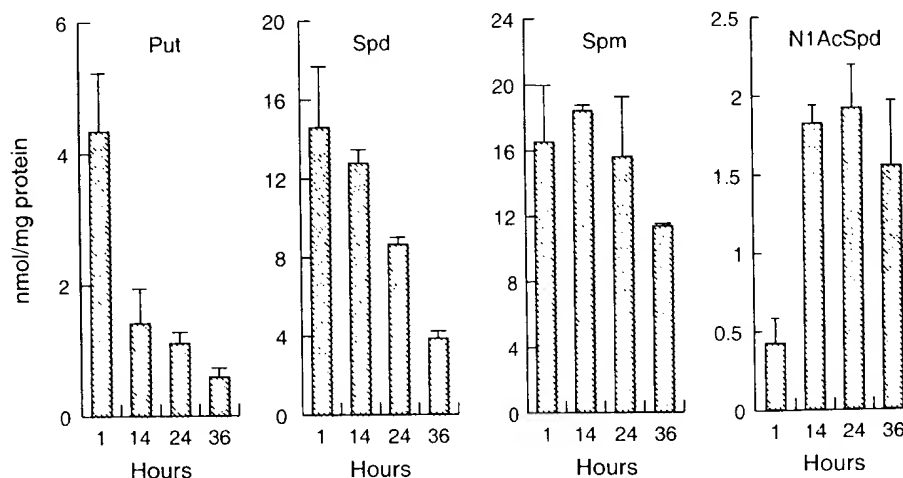
Fig. 1. MDL-72,527 alters polyamine pools in myeloid cells. 32D.3 cells cultured in replete growth medium (RPMI 1640/20 units/ml IL-3 and 10% dialyzed FBS) were cultured in the presence of 150 μM MDL-72,527. At the indicated intervals, 10^7 cells were collected and analyzed for their concentrations of intracellular Put, Spd, Spm, and N^1-acetyl Spd. N^1-acetylated Spm was not detected (negative data not shown). Results shown are taken from two separate HPLC determinations.
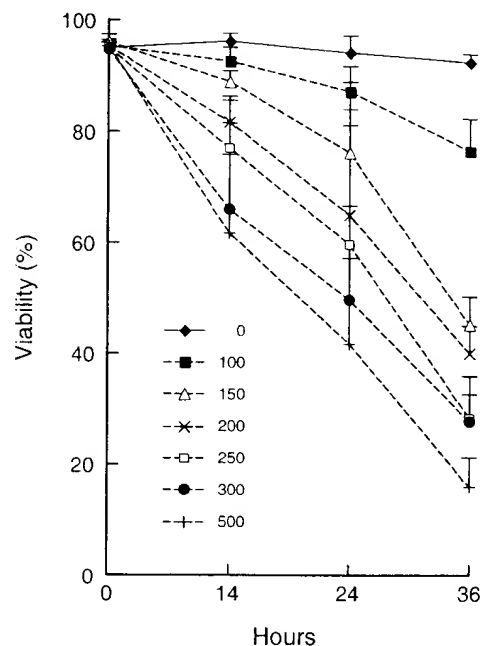
32D.3 cells with increasing doses of MDL-72,527. Surprisingly, even at doses lower than those known to be protective for other cell types (*i.e.,* 150 μM; see Ref. 5), MDL-72,527 induced the rapid death of these myeloid progenitors, as monitored by the uptake of the dye trypan blue, despite the presence of the strong survival factors IL-3 and serum (Fig. 2A). Toxic effects of this PAO inhibitor were also observed after treatment of another immortal IL-3-dependent myeloid cell line, FDC-P1 (Fig. 2B). Rates of cell death induced by MDL-72,527 were dose dependent (Fig. 2A) and with time led to the death of all of the cells present in the culture (data not shown). A trivial explanation for this toxicity could have been that the MDL-72,527 inhibitor, being a polyamine analogue (15), was directly oxidized by serum amine oxidase, which also generates H_2O_2 (27), and this activity is toxic to 32D.3 cells when cultured in Spd or Spm (Ref. 19 and data not shown). However, cotreatment of the cells with aminoguanidine, a potent inhibitor of serum amine oxidase, did not block MDL-72,527-induced death, and cells were comparably sensitive to this inhibitor when cultured in normal FBS (data not shown). Thus, treatment of cells with MDL-72,527 overrides the protective functions of the survival factors IL-3 and serum in immortal myeloid progenitors, and this cell death is not influenced by polyamines or polyamine-directed enzymes present in serum.

Cytokines are continuously required to suppress apoptosis in myeloid progenitors (18, 28). Overexpression of oncoproteins such as c-Myc or E2F-1, which function as master regulators of the cell cycle, overrides the protective effects of survival factors, including IL-3 and serum, to induce apoptosis (18, 29–31). However, treatment of these myeloid cells with 150 μM MDL-72,527 had no effect on c-Myc or E2F-1 protein levels and did not lead to overt changes in their cell cycle profiles (data not shown). Therefore, MDL-72,257 overrides survival factors to induce apoptosis without affecting the cell cycle or levels of cell cycle regulators, such as c-Myc or E2F-1.

MDL-72,527-induced Apoptosis Is Independent of Changes in Polyamine Pools. Measurement of polyamine pools (Fig. 1) demonstrated that MDL-72,527-reduced levels of Put and Spd and increased levels of N^1-acetyl-Spd. To determine whether the deleterious effects of MDL-72,527 were, indeed, due to decreases in Put and Spd pools, we tested the ability of exogenously added Put or Spd to block MDL-72,527-induced cell death. The addition of either polyamine failed to block inhibitor-induced cell deaths (Fig. 3A), despite the fact that Put is known to effectively reverse DFMO-induced G_1 arrest in these same cells (19). Similarly, 32D.3 cells that overproduce Put due to engineered overexpression of ODC (19) also died when treated with MDL-72,527 (Fig. 3B). Finally, pretreatment of 32D.3 cells with 5 mM DFMO, which markedly decreases levels of Put and Spd, and leads to cell cycle arrest (Ref. 19 and data not shown), actually increased the sensitivity of 32D.3 cells to MDL-72,527 (Fig. 3C). This finding is consistent with the observed synergism of these inhibitors in tumor cells (Refs. 13 and 16 and see below). Therefore, MDL-72,527-induced cell death seemed to occur independently of overt alterations in steady-state pools of polyamines.

MDL-72,527-induced Apoptosis Is Associated with Down-Regulation of the Cell Death Antagonist Bcl-X_L, and the Activation of Caspase-3. To confirm that the myeloid cell death observed after treatment with MDL-72,527 was, indeed, apoptotic, we assessed the integrity of genomic DNA by agarose gel electrophoresis (Fig. 4A) and Annexin V/propidium iodide FACS staining (Fig. 4B). Apoptosis is usually accompanied by internucleosomal nicking of genomic DNA that results in a typical "laddering" of DNA (32, 33) and the flipping of phosphatidylserine from the inner to the outer leaflet of the cell membrane (23). Both of these hallmarks were clearly evident in 32D.3 cells cultured in IL-3 after treatment with MDL-72,527. Cleavage of genomic DNA was almost complete (*i.e.,* DNA that remained in the
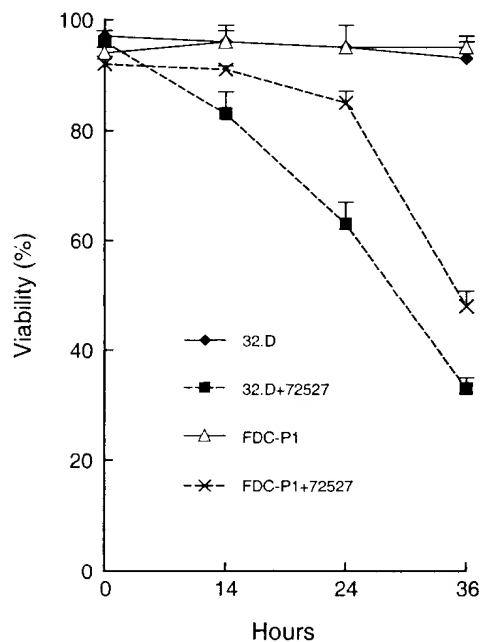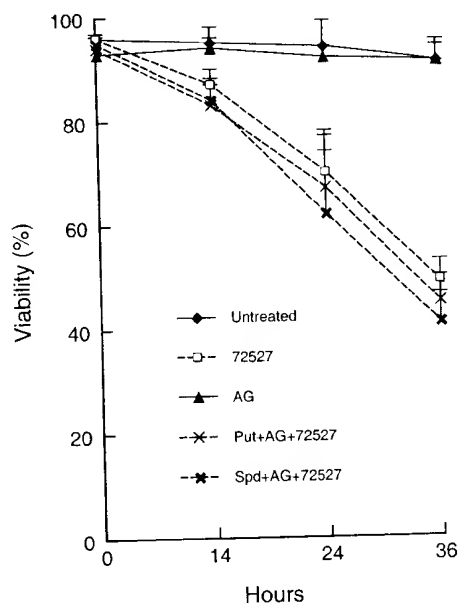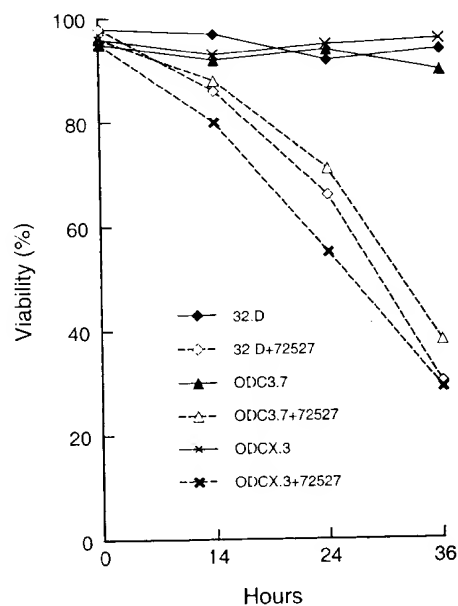
A.

B.

Fig. 2. The PAO inhibitor MDL-72,527 overrides the survival functions of IL-3 and serum. A, IL-3 dependent 32D.3 cells growing in RPMI 1640 containing IL-3 (20 units/ml) and 10% dialyzed FBS (polyamine-depleted FBS) were treated with the indicated concentrations of the PAO inhibitor MDL-72,527. The percentage of viable cells was quantitated by trypan blue dye exclusion at indicated intervals. The values shown are averages from three separate experiments. B, MDL-72,527 also compromises the survival of IL-3-dependent FDC-P1 myeloid cells. FDC-P1 cells were cultured in RPMI 1640 supplemented with IL-3 (20 units/ml) and 10% dialyzed FBS. The percentage of viable cells was calculated at the indicated intervals by trypan blue dye exclusion. Results shown are representative of three independent experiments.

well) by 24 h after treatment with 150 μM MDL-72,527 (Fig. 4A). Furthermore, there were obvious early apoptotic cells, as judged by their Annexin-V-positive, PI-negative phenotype by FACS, soon after the addition of MDL-72,527 (Fig. 4B). By FACS analyses, it was also
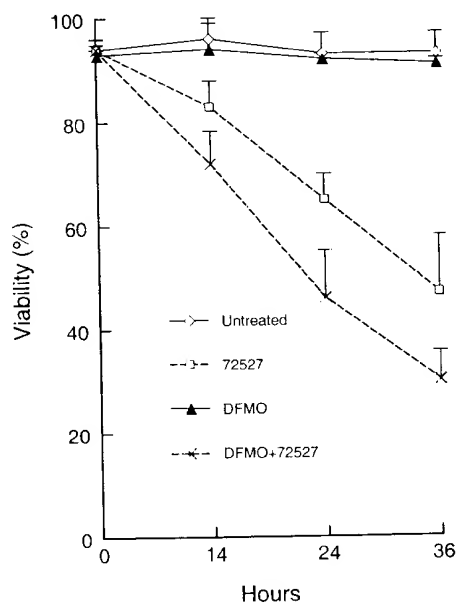
A.

B.

C.

Fig. 3. MDL-72,527-induced cell death is independent of polyamine levels. *A*, Put and Spd fail to rescue cell death induced by 150 μM MDL-72,527. 32D.3 cells carried in RPMI 1640/20 units/ml IL-3 and 10% dialyzed FBS were left untreated or were treated with 1 mM Put or Spd in the presence of aminoguanidine for 36 h and assessed for viability, as described in the legend to Fig. 2. *B*, overexpression of ODC does not attenuate MDL-72,527-induced apoptosis. 32D.3 parental cells and 32D.3 derived clones that overexpress ODC (and have elevated levels of Put; Ref. 19) carried in RPMI 1640/20 units/ml IL-3 and 10% dialyzed FBS were treated with or without 150 μM MDL-72,527. At the indicated intervals, cell viability was assessed as described previously. *C*, cells depleted of polyamines with DFMO are still sensitive to MDL-72,527. 32D.3 cells carried in RPMI 1640/20 units/ml IL-3 and 10% dialyzed FBS were left untreated or were treated with 5 mM DFMO. After 48 h, a portion of the cultures was then treated with 150 μM MDL-72,527, and cell viability was determined at the indicated intervals. Results shown are representative of three independent experiments.

evident that MDL-72,527-treated cells had substantial increases in mean cell size (see top panels, Fig. 4*B*, and see Figs. 7 and 8).

We also investigated the effects of MDL-72,527 on the steady-state levels of the Bcl-2 family of apoptotic regulators that either inhibit (*e.g.*, Bcl-2 and Bcl-X$_L$) or induce (*e.g.*, Bax and Bad) apoptosis (34). In myeloid progenitors, cytokines suppress apoptosis through the selective up-regulation of the cell death antagonist Bcl-X$_L$ (21). Interestingly, treatment of 32D.3 myeloid cells growing in IL-3 with the inhibitor induced the selective down-regulation of Bcl-X$_L$ protein, as detected by immunoblot analyses (Fig. 5*A*), suggesting that the induction of apoptosis by MDL-72,527 was linked to its ability to inhibit IL-3-dependent signaling pathways required to sustain Bcl-X$_L$ expression. By contrast, MDL-72,527 had no effect on the steady-

state levels of other Bcl-2 family members, including the antiapoptotic protein Bcl-2 and the proapoptotic proteins Bax and Bad (Fig. 5*A*).

Antiapoptotic Bcl-2 family members suppress apoptosis by inhibiting the activation of a cascade of caspases (*c*ysteine-dependent, *asp*artate-specific prote*ases;* 35). In most cell death pathways, the final enzyme of this cascade is caspase-3, which is activated after proteolytic cleavage of a proenzyme form. Activated caspase-3, in turn, cleaves specific proteins that are required to maintain cellular integrity, for example PARP (35). Immunoblot analyses demonstrated that both the pro-form of caspase-3 and PARP became cleaved in MDL-72,527-treated cells and that these events coincided with the death of these cells (Fig. 5*B*). Thus, by these biochemical criteria, MDL-72,527 induces cell death that is typical of apoptosis, and this
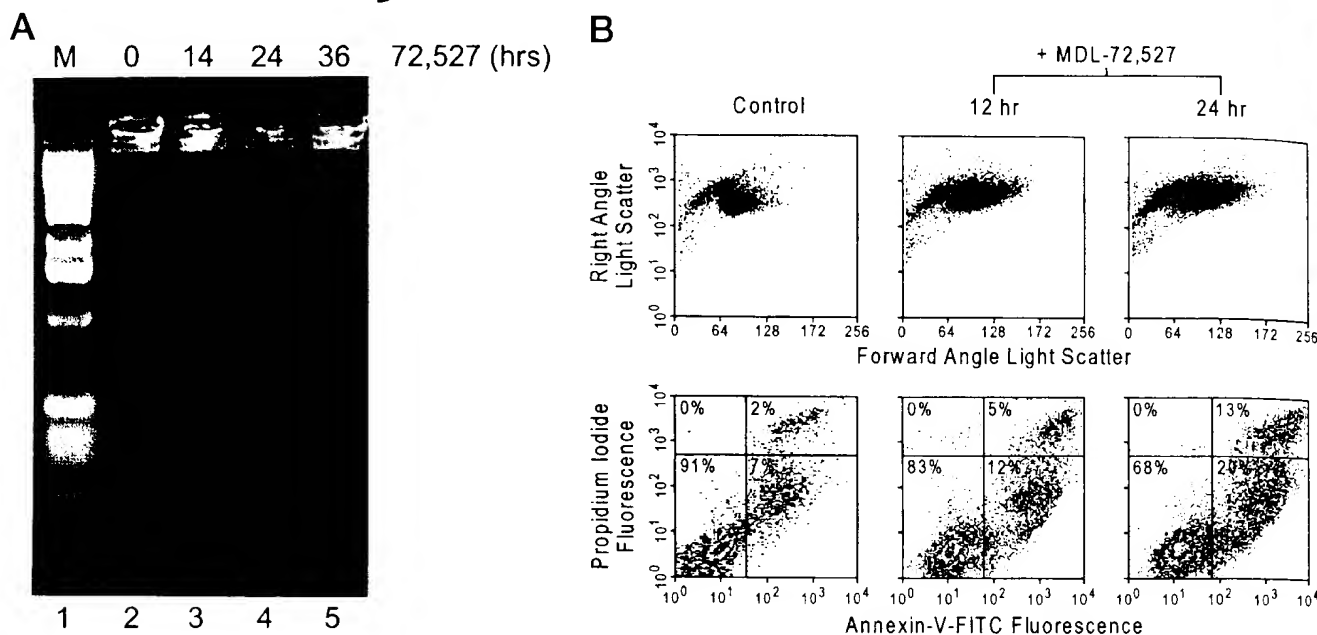
Fig. 4. The PAO inhibitor MDL-72,527 induces myeloid cell apoptosis. 32D.3 myeloid cells in RPMI 1640/20 units/ml IL-3 and 10% dialyzed FBS were cultured in the presence of 150 μM MDL-72,527 and characterized for apoptosis by analysis of genomic DNA (A; ladders) and Annexin V staining (B). A, agarose gel electrophoretic analysis of genomic DNA isolated from 10^6 32D.3 cells for indicated intervals in the presence (Lanes 3, 4, and 5) or absence (Lane 2) of 150 μM MDL-72,527. M, molecular marker (Life Technologies, Inc.). B, 32D.3 myeloid cells in RPMI 1640/20 units/ml IL-3 and 10% dialyzed FBS were cultured in the presence of 150 μM MDL-72,527 for the indicated intervals, and Annexin V assays performed as described in "Materials and Methods." The lower right quadrant indicates the percentage of early apoptotic cells (PI low, Annexin V bright), whereas late apoptotic cells (upper right) are both PI and Annexin V bright. Note that MDL-72,527 treatment also caused evident increases in mean cell size (top panels), consistent with the appearance of large vacuoles in MDL-72,527 treated cells (see Figs. 7–9).

activity is associated with the down-regulation of the cell death antagonist Bcl-X_L.

MDL-72,527 Overrides the Protective Functions of the Cell Death Antagonists Bcl-2 and Bcl-X_L and That of the Caspase Inhibitor zVAD-fmk. The selective down-regulation of Bcl-X_L in cells treated with MDL-72,527 suggested that overexpression of Bcl-X_L or Bcl-2 might block MDL-72,527-induced cell death. We, therefore, assessed the effects of this inhibitor in 32D.3 derivatives engineered to overexpress Bcl-2 or Bcl-X_L. These cells display remarkable resistance to apoptosis after the depletion of IL-3 (20, 21) or treatment with chemotherapeutic drugs (20). Although the rates of death of cells overexpressing Bcl-2 or Bcl-X_L were protracted relative to vector-only controls, these cells also ultimately succumbed to MDL-72527, despite the fact that they maintained elevated levels of exogenous Bcl-2 or Bcl-X_L and were cultured in replete medium having a full complement of survival factors (Fig. 6A and data not shown). Moreover, treatment of cells with the universal caspase inhibitor zVAD-fmk (35) failed to block MDL-72,527-induced cell death (Fig. 6B), although zVAD-fmk delayed the rate of apoptosis of 32D.3 myeloid cells after IL-3 withdrawal (Fig. 6B). Thus, MDL-72,527 treatment results in the selective down-regulation of Bcl-X_L and activation of caspase-3, yet strategies directed to override these effects of the PAO inhibitor failed to block MDL-72,527-induced cell death.

MDL-72,527-induced Apoptosis Is Due to Lysosomotropic Effects. Morphological examination of cytospins of MDL-72,527-treated 32D.3 and FDC-P1 myeloid cells revealed the rapid appearance of remarkably large cytosolic vacuoles (data shown for 32D.3 cells in Figs. 7 and 8). Vacuoles appeared within 4 h of MDL-72,527 treatment and, with time, coalesced to form even larger vacuoles that approached a volume of at least half the cell. By both electron (Fig. 7) and light microscopy (Fig. 8), vacuole formation preceded other changes associated with apoptosis, including condensation of chromatin, but, with time, these more typical markers of apoptosis were

also evident. Ultrastructural studies revealed that these vacuoles were of lysosomal origin, and treatment of these myeloid cells with chloroquine, a known lysosomotropic agent (36–38), induced the appearance of similar vacuoles (data not shown). With time, the membranes containing these expanding lysosomes broke down and the vacuoles appeared to overtake key cellular organelles, including mitochondria, and eventually destroyed cellular architecture (Fig. 7). However, if myeloid cells were transiently exposed to MDL-72,527 and then placed in replete medium free of the inhibitor existing vacuoles shrunk (Fig. 8), suggesting that cells can either export or metabolize MDL-72,527 and that early vacuolation is reversible.

Accumulation of MDL-72,527 in Immortal Myeloid Progenitors and Leukemia Cells Correlates with Its Cytotoxic and Lysosomotropic Effects, but not in Primary Myeloid Progenitors. The rapid, but reversible, appearance of large lysosomally derived vacuoles in MDL-72,527-treated 32D.3 myeloid cells suggested that this response might directly correlate with increased intracellular accumulation of this polyamine analogue via a lysosomotropic mechanism of drug entrapment (36–38). Indeed, direct measurements of the MDL-72,527 inhibitor by HPLC of treated 32D.3 cells revealed high intracellular concentrations of MDL-72,527 (Fig. 9A). In fact, on a molar basis MDL-72,527 levels were significantly higher than levels of endogenous polyamines within 24 h in 32D.3 cells (compare Fig. 1 and Fig. 9A). Thus, the ability of this inhibitor to induce lysosomotropic effects and apoptosis of 32D.3 cells correlated with the abundant intracellular accumulation of MDL-72,527.

Given the profound sensitivity of immortal myeloid progenitors to MDL-72,527 (Fig. 2), we addressed whether this response was selective by analyzing the sensitivity of primary myeloid cells, derived from day 15 embryonic murine fetal liver (21), and cultured murine and human leukemia cell lines. In contrast to immortal progenitor cell lines, the survival (and growth) of primary murine myeloid progenitors was not appreciably affected by even very high doses of the inhibitor (Fig. 9B and data not shown). However, certain leukemia
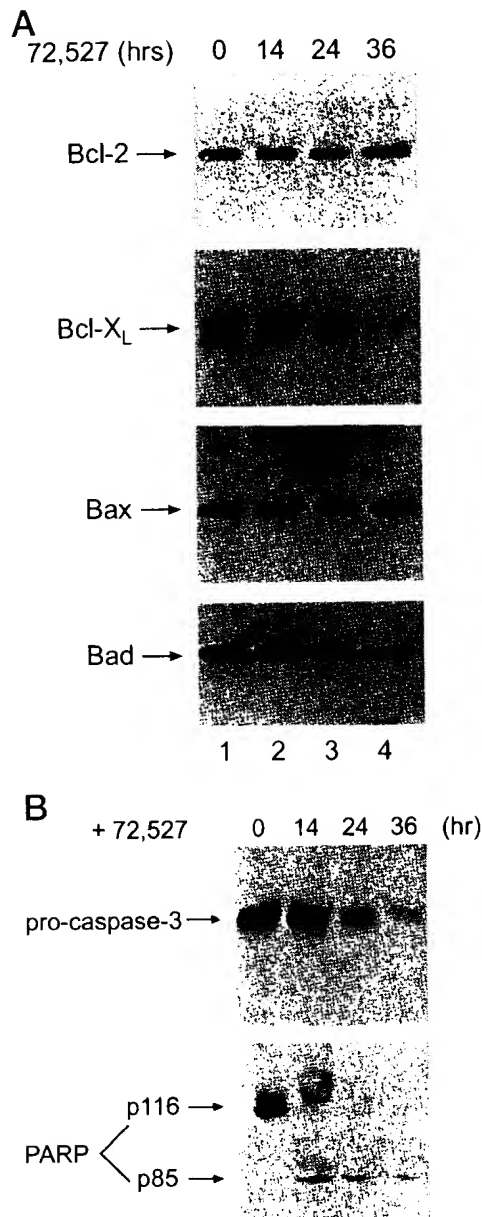
A

72,527 (hrs)   0   14   24   36

Bcl-2 →

Bcl-X$_L$ →

Bax →

Bad →

1   2   3   4

B

+ 72,527   0   14   24   36   (hr)

pro-caspase-3 →

PARP ⟨ p116 →
       p85 →

Fig. 5. Apoptosis induced by MDL-72,527 is associated with down-regulation of the cell death antagonist Bcl-X$_L$ and activation of caspase-3. *A*, 32D-3 myeloid cells carried in RPMI 1640/20 units/ml IL-3 and 10% FBS were cultured in the presence or absence of 150 μM MDL-72,527. Cells (5 × 10⁶) were harvested at the indicated intervals, and 50 μg of whole cell extract were analyzed by immunoblot analyses with antibodies that detect murine Bcl-2, Bcl-X$_L$, Bax, and Bad. *B*, Cells (5 × 10⁶) were harvested at the indicated intervals, and 50 μg of whole cell extract were analyzed by immunoblot analyses with antibodies that detect murine procaspase-3 and PARP. Results shown are representative of two independent experiments.

cells (*e.g.*, human HL-60 myeloid leukemia cells and murine L1210 leukemia cells) were exquisitely sensitive to the deleterious effects of MDL-72,527 (Fig. 9*B*), whereas others (*e.g.*, human U937 and Molt3 leukemia cells) were less sensitive to the inhibitor than immortal 32D.3 myeloid cells (Fig. 9*B*). Given that intracellular levels of the PAO inhibitor in 32D.3 cells seemed to correlate with its lysosomotropic effects and toxicity, we also measured the intracellular accumulation of MDL-72,527 and its lysosomotropic effects in this panel of malignant hematopoietic cells. Surprisingly, the ability of the inhibitor to induce lysosomotropic effects and subsequent cell death did not strictly correlate with the intracellular accumulation of the inhibitor. Primary myeloid cells that were resistant to the deleterious

effects of MDL-72,527 accumulated abundant quantities of MDL-72,527 without obvious lysosomotropic effects (Fig. 9*C*). Therefore, intracellular accumulation of MDL-72,527 is likely necessary, but not sufficient, for the observed lysosomotropic effects of this inhibitor.

A

Viability (%)

Hours

- ◆ 32 D
- ■ 32 D+72527
- △ Bcl-2
- ✳ Bcl-2+72527
- ✶ Bcl-X$_L$
- ○ Bcl-X$_L$+72527

B

Viability (%)

Hours

- ◆ Control
- ▲ 72527
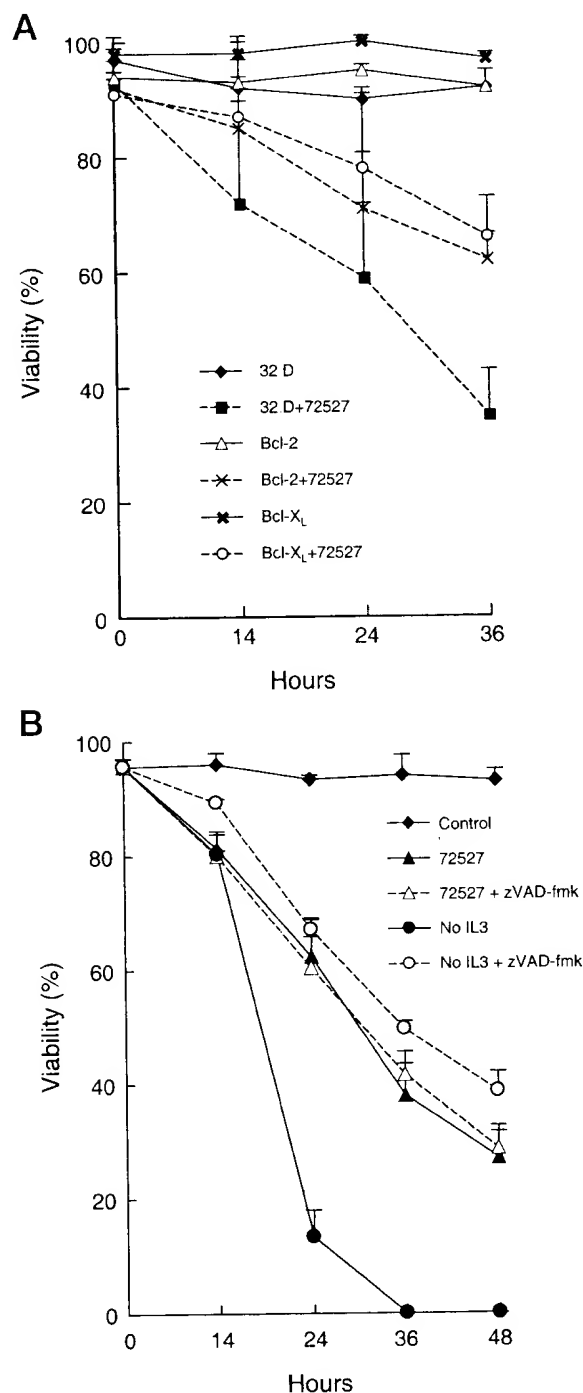- △ 72527 + zVAD-fmk
- ● No IL3
- ○ No IL3 + zVAD-fmk

Fig. 6. Overexpression of Bcl-XL and Bcl-2 or treatment with the caspase inhibitor zVAD-fmk delays, but fails to inhibit, MDL-72,527-induced apoptosis. *A*, 32D.3 parental cells and 32D.3-derived clones that overexpress Bcl-2 or Bcl-XL (20, 21) cultured in replete medium (RPMI 1640/20 units/ml IL-3, 10% FBS, and G418) were left untreated or treated with 150 μM MDL-72,527, and cell viability was assessed at the indicated intervals as described previously. Results shown are representative of three independent experiments. *B*, 32D.3 cells were pretreated with the universal caspase inhibitor zVAD-fmk (400 μM) for 1 h and then left untreated or treated with 150 μM MDL-72,527. At the indicated intervals, cell viability was determined by trypan blue dye exclusion. Results shown are representative of three separate experiments.
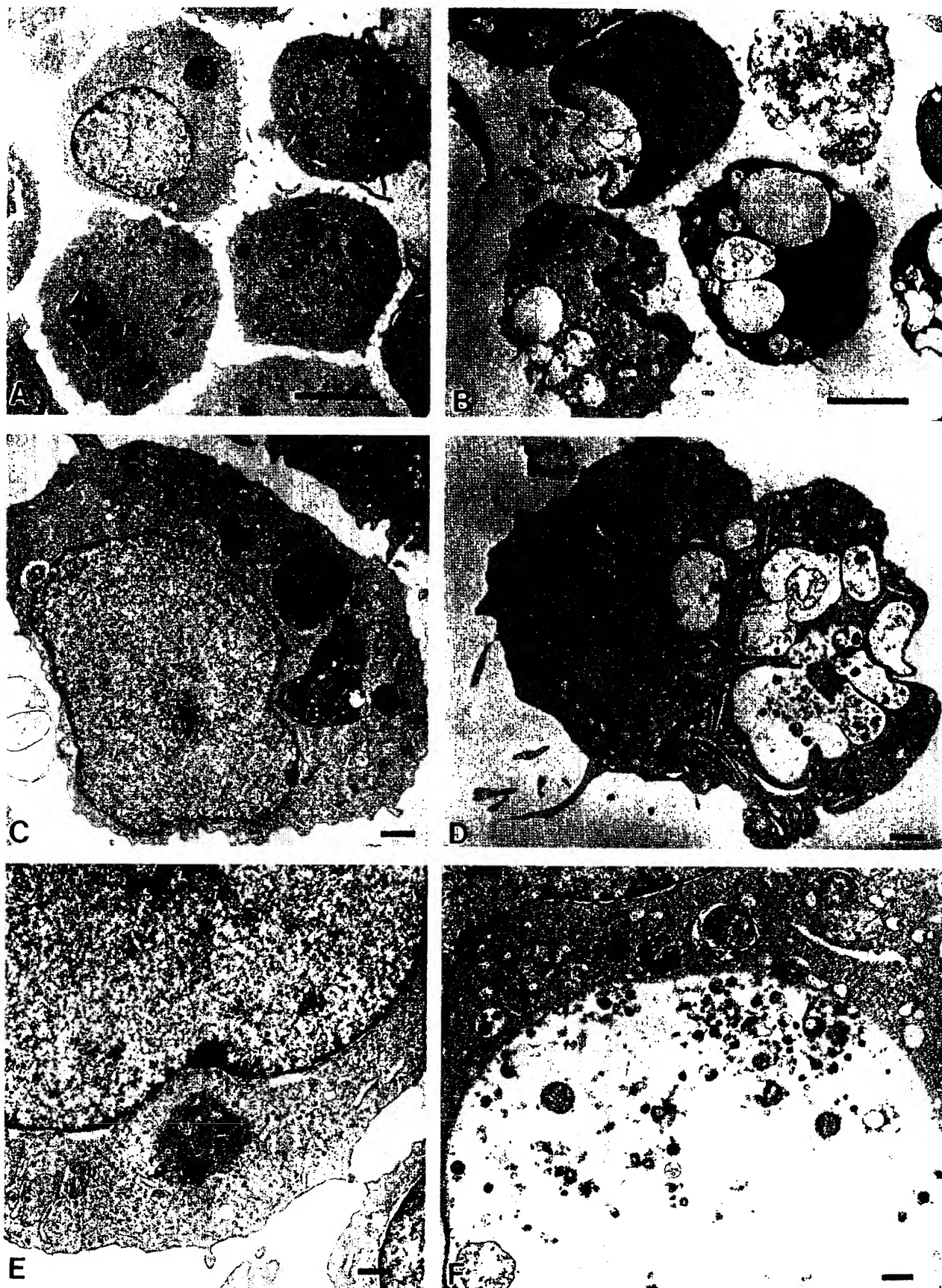
Fig 7 MDL-72,527 induced apoptosis is associated with lysosomotropic effects. Transmission electron microscopy of control 32D.3 cells (left, A, C, and E) and MDL-72,527-treated cells (B, D, and F) Note the prominent vacuoles (swollen lysosomes) that appear in MDL-72,527-treated cells (B, D, and F) Cells shown were treated with the analogue for 14 h, and magnifications were ×4,500 (A and B), ×12,500 (C and D), and ×60,000 (E and F) Bars in A and B equal 10 μм and in C-F equal 0.5 μм. Note the dissociation of lysosomal membrane and the presence of mitochondria and other organelles within these vacuoles (D and F).
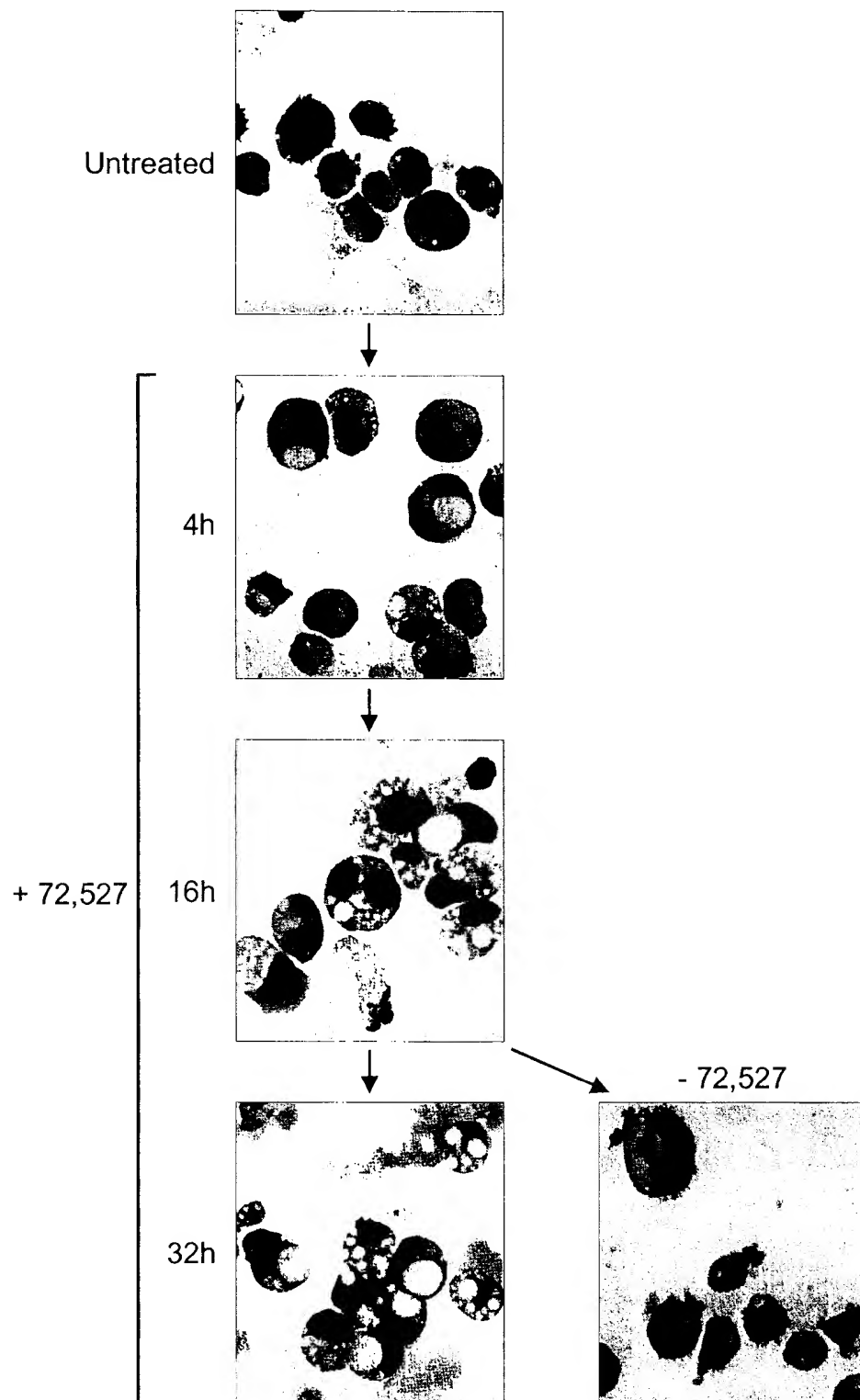
Fig. 8. Lysosomotropic effects of MDL-72,527 are reversible in treated myeloid cells. 32D.3 cells cultured in replete growth medium were treated with 150 μM MDL-72,527 for the indicated intervals. After 16 h, cells in the inhibitor were washed and then put back into fresh medium with or without MDL-72,527 for an additional 16 h. Magnifications (×100) of cytospins of Wright-Geimsa-stained cells are shown. Note the prominent vacuoles that appear in MDL-72,527-treated cells. Representative fields of cells are shown. Similar vacuoles were observed in FDC-P1.2 myeloid cells treated with 150 μM MDL-72,527 (data not shown).

Combination of MDL-72,527 with the ODC Inhibitor DFMO Induces Leukemia Cell Death. A dose-limiting toxicity of many chemotherapeutic regimens is hematopoietic deficits such as neutropenia. The uptake of polyamines in most cell types is increased after the exposure of cells to the ODC inhibitor DFMO, in efforts to recoup diminishing pools of intracellular Put and Spd. Given the proposed combined use of MDL-72,527 and DFMO in some applications (12, 16), we evaluated the combined effects of these inhibitors in primary and immortal hematopoietic cells and in leukemia cells. In all cells treatment with DFMO alone had virtually no effect on cell survival (Fig 9B), although, as predicted (9, 19), it did block cell growth and led to predictable decreases in Put and Spd pools in all cells tested (data not shown). Treatment of primary myeloid cells with both DFMO and MDL-72,527 did not enhance accumulation of MDL-72,527 in these cells (Fig. 9A) and did not lead to the formation of lysosomal vacuoles (Fig. 9C). By contrast, the combination of DFMO
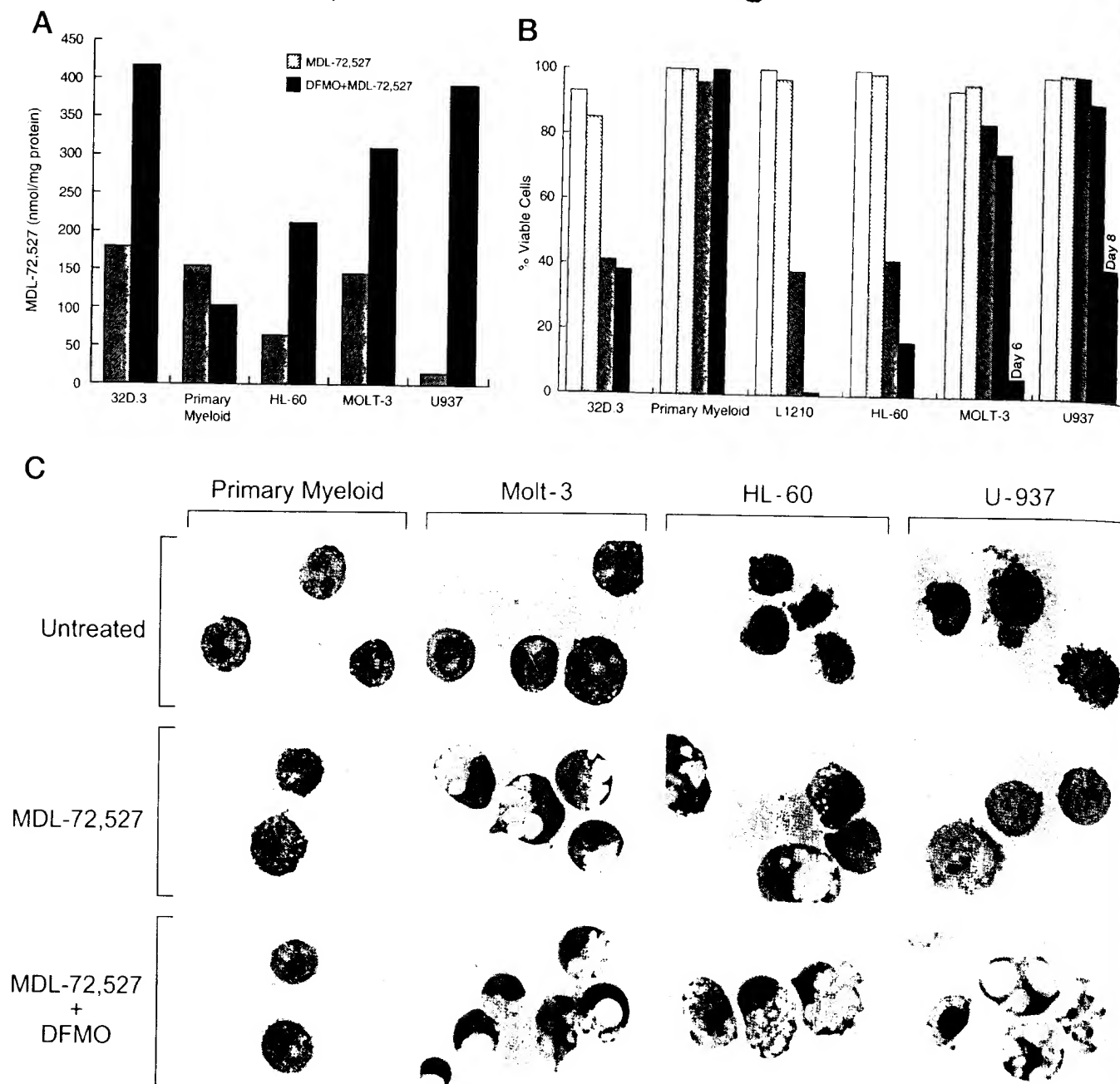
Fig. 9. Intracellular accumulation of MDL-72,527 is associated with selective lysosomotropic and cytotoxic effects in leukemia cells and is potentiated by the ODC inhibitor DFMO. *A*, intracellular accumulation of MDL-72,527 +/− DFMO in immortal (32D.3) and primary myeloid cells and in murine and human leukemia cells (L1210, HL-60, MOLT-3, and U937 leukemia cells). Cells cultured in replete growth medium were cultured in the presence or absence of 5 mM DFMO for 48 h and then treated with 150 μM (32D.3 cells) or 300 μM (all others) MDL-72,527. At 24 h cells were collected and analyzed for their concentrations of MDL-72,527 by HPLC. *B*, DFMO treatment potentiates MDL-72,527-induced cytotoxicity. Twenty-four h after adding MDL-72,527, the percentage of viable cells was calculated by trypan blue dye exclusion. For U937 and Molt3 cells, results are also shown for days 6 or 8 of culture in MDL-72,527 + DFMO, respectively. All leukemia cells, but not primary myeloid cells, receiving both DFMO and MDL-72,527 ultimately died. Results shown are representative of four independent experiments □, untreated; ▨, +DFMO; ▧, +MDL-72,527; ■, +DFMO+MDL-72,527. *C*, DFMO potentiates MDL-72,527-induced lysosomotropic effects in leukemia cells. The indicated cells were cultured in MDL-72,527 +/− DFMO, and cell morphology of cytospins was analyzed by Wright-Geimsa staining. The magnification shown is ×100. Results shown are representative of two independent experiments.

and MDL-72,527 markedly enhanced the formation of lysosomal vacuoles in all leukemia cells tested (Fig. 9*C*). With time, DFMO also exacerbated the cytotoxic effects of MDL-72,527 such that all leukemia cells ultimately died, but remained without cytotoxic effects on primary myeloid cells (Fig. 9*B* and data not shown). In all leukemia cells the deleterious combined effects of DFMO and MDL-72,527 were associated with a marked increase in intracellular accumulation of MDL-72,527, as measured by HPLC (Fig. 9*A*). Moreover, the deleterious effects of DFMO when combined with MDL-72,527 in malignant cells were fully reversed when cells were cultured with

exogenous Put (data not shown). Thus, malignant but not primary hematopoietic cells are sensitive to the combined actions of DFMO and MDL-72,527, and this is associated with the selective lysosomotropic effects of the inhibitor in these transformed cells.

DISCUSSION

Polyamine functions are presumed to be diverse and are known to be required for cell growth and survival (1, 2). Thus, disruption of polyamine homeostasis would be predicted to have cytostatic or

cytotoxic consequences. The biological effects of inhibitors that specifically target ODC or SAMDC, or of agonists that induce SSAT, amply support this notion (3–9, 11, 14). By contrast, inhibition of PAO, the oxidative component of the catabolic polyamine salvage pathway, has not been appreciably associated with cytostatic or cytotoxic affect (14, 17). Indeed, MDL-72,527 treatment has been reported to block the toxic effects of SSAT agonists in CHO cells and non-small cell lung carcinoma cells, apparently by inhibiting the generation of H_2O_2 (5, 6). It was, thus, surprising to find that MDL-72,527 is a potent inducer of apoptosis in immortal and malignant hematopoietic cells. Moreover, this analogue effectively overrides the series of cellular defenses that block this suicide program, including the functions of survival factors and antiapoptotic Bcl-2 proteins, and can not be blocked by a broad spectrum caspase inhibitor. MDL-72,527 had predictable lowering effects on steady-state levels of Put and Spd and on the formation of N^1-acetylated-Spd, but a series of genetic and biochemical experiments demonstrated that cell death after treatment with this analogue was independent of its effects on polyamine pools. Rather, we have found a selective cytotoxicity of the PAO inhibitor MDL-72,527 toward immortal or malignant hematopoietic cells and that this seems to be related to inherent lysosomotropic properties of the drug.

Although not previously reported for MDL-72,527, lysosomotropic-associated effects for the SSAT agonist DENSPM and the polyamine analogue 2–5-diamine2–3-hexyne have also been described (39, 40). The vacuoles that arise in MDL-72,527-treated hematopoietic cells are lysosomal in origin based on their structure under electron microscopy, the ability to take up lysosome-specific dyes (40 and data not shown), and of their similarity to vacuoles induced by chloroquine (36–38). With time, these vacuoles swell to remarkable size, and this likely leads to a failure of the lysosomal membrane and subsequent ingestion of the cell from within (Fig. 7). An underlying reason that MDL-72,527 has these lysosomotropic effects may, in part, be due to its property as a weak base, which targets the lysosome, because artificially altering the cellular pH ablates its lysosomotropic and toxic effects.[4] Analogous findings have been reported for an amine-based lysosomotropic detergent, which directly influences intralysosomal pH and kills cells by disrupting the integrity of the lysosome (41). However, this seems to be only one contributing factor because primary myeloid cells are resistant to MDL-72,527-induced lysosomotropic and cytotoxic effects, but still accumulate high levels of MDL-72,527 (Fig. 9A). This apparent lysosomal difference between normal and malignant myeloid cell warrants further investigation.

Given the effects of MDL-72,527 on lysosome structure and leukemia cell death, we have tested other polyamine analogues and inhibitors for comparable effects, including the ODC inhibitor DFMO, the SAMDC inhibitor CGP-48664 (4-amidinoindan-1–2′-amidinohydrazone; Ref. 8), and the SSAT agonist DENSPM (40). Only MDL-72,527 induced lysosomotropic effects in immortal 32D.3 and FDC-P1.2 myeloid cells, despite the fact that DENSPM has these effects when added to Chinese hamster ovary cells that overexpress SAMDC (40). Furthermore, addition of exogenous Put or Spd failed to reveal lysosomotropic effects (data not shown). Thus, the induction of lysosomal vacuoles, in at least immortal hematopoietic cells, seems selective for only some polyamine analogues, such as MDL-72,527. Formation of the lysosomal vacuoles is tightly associated with cell death because both vacuole formation and apoptosis were reversible if drug was removed from transiently exposed cells (Fig. 8). Thus, the eventual death of immortal or malignant hematopoietic cells seems due to the combined effects of MDL-72,527 intracellular accumulation (Fig. 9) and its associated lysosomotropic effects. Interestingly, intracellular accumulation of this PAO inhibitor does not necessarily equate into lysosomotropic effects because primary cells accumulate prodigious amounts of the inhibitor without appreciable deleterious effects. Alternatively, we can not exclude that the more modest accumulation of N^1-acetylated Spd observed in MDL-72,527-treated immortal myeloid cells (Fig. 1) also contributes to the formation of lysosomal vacuoles and eventual cell death, and this issue also warrants further investigation.

There are clearly several contributing factors to the observed lysosomotropic effects of the PAO inhibitor MDL-72,527. First, uptake, export, and/or metabolism of the inhibitor are important issues that seem to be cell context dependent because parallel measurements of MDL-72,527-resistant versus -sensitive cells demonstrated that intracellular accumulation of the inhibitor seems necessary, but not sufficient, for the formation of lysosomal vacuoles and cytotoxicity. Accumulation of the inhibitor does not seem to be due to a specialized intrinsic polyamine import or export system present in these immortal or malignant hematopoietic cells because polyamines or other polyamine analogues do not have this effect on these cells. Second, there are also apparently context-specific effects in regard to the effects of polyamine analogues on lysosome functions. For example, DENSPM induces lysosomotropic effects in CHO cells that overexpress SAMDC due to gene amplification (40), but DENSPM accumulates rather poorly in myeloid progenitors and never induces vacuoles or cytostatic effects, even when polyamine import is increased by cotreatment with DFMO.[5] Also, the polyamine analogue 2–5-diamine2–3-hexyne induces lysosomal vacuoles and toxicity in L1210 leukemia cells, but not all cells types (37). Finally, the cytotoxicity of polyamine analogues in hematopoietic cells does not necessarily correlate with the formation of lysosomal vacuoles. For example, the SAMDC inhibitor CGP-48664 compromises myeloid cell survival without lysosomal effects.[6]

The findings that MDL-72,527 can alone compromise the survival of some leukemia cells suggest that it may, if properly delivered, have some therapeutic benefit. In this regard, it is interesting that the selective toxicity of MDL-72,527 is potentiated by the treatment of leukemia cells with DFMO, which induces polyamine uptake in most cells (42). This finding may at least, in part, explain the increased efficacy of these two inhibitors in a therapeutic context (11, 12). It is also quite compelling that MDL-72,527 overrides the functions of antiapoptotic Bcl-2 proteins, and that this is also associated with the down-regulation of Bcl-X$_L$. The latter finding is particularly noteworthy because Bcl-X$_L$ has been identified as a key target of cytokine-mediated survival pathways and is also selectively activated in hematopoietic malignancies (19). The fact that MDL-72,527 suppresses Bcl-X$_L$ suggests that polyamines may influence survival-signaling pathways. However, because enforced overexpression of Bcl-X$_L$ failed to block the deleterious effects of this analogue, it seems death due to lysosomotropic effects is a mortal insult.

ACKNOWLEDGMENTS

[4] H. Dai and J. L. Cleveland, unpublished observations.

[5] Unpublished observations.

[6] J. L. Cleveland, unpublished results.

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